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Enabling Clearance Predictions to Emerge from In Silico Actions of Quasi-Autonomous Hepatocyte Components[S]

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ABSTRACT:

We demonstrate the feasibility of using in silico hepatocyte cultures (ISHCs) to provide predictions of the intrinsic clearance (CL) of compounds in hepatocyte cultures. We compare results with predictions obtained using a multiple linear regression method. Our expectation is that the method can be extended to predict in vivo clearance of new compounds in humans. Within ISHCs, mobile "compounds" carry information describing referent compound properties. We used an iterative refinement protocol for ISHC refinement and development of parameterization methods. Quasi-autonomous "hepatocytes" and their components (including "transporters" and "enzymes") use a small, event-specific subset of compound properties to decide how to interact with encountered compounds each simulation cycle. The probability of occurrence for each event is specified by a rule that uses a subset of compound properties known to influence that event in vitro. ISHC experiments mimic in vitro counterparts. In silico clearance is measured the same as in vitro clearance and is used to predict a corresponding CL value. For 39 of 73 compounds having calculated CL S.D.s, 79% of ISHC predictions and 23% of regression predictions were within CL ± 2 S.D. For all 73 compounds, 38% of ISHC predictions and 32% of regression predictions were within a factor of 2 of the referent CL values. ISHC details during simulations stand as a mechanistic hypothesis of how clearance phenomena emerge during in vitro experiments.

Introduction

Significant progress has been made in using in vitro data to predict in vivo hepatic clearance in humans. Further improvement is needed (Baker and Parton, 2007; Stringer et al., 2008; Hallifax et al., 2010; Huang et al., 2010; Rostami-Hodjegan, 2010), which opens the door for exploring alternative strategies, such as methods that use mechanistic knowledge instantiated in models built and validated using object- and agent-oriented, discrete event methods of modeling and simulation (Zeigler, 1984; Zeigler et al., 2000; Hunt et al., 2009). A precondition for achieving in vivo models of that type is to first have simpler models designed to provide acceptable predictions of intrinsic clearance (CL) in hepatocyte cultures, and that is the focus of this research. Early-stage reports support feasibility (Liu and Hunt, 2006; Sheikh-Bahaei and Hunt, 2006; Sheikh-Bahaei et al., 2006). However, they focused on six or fewer compounds. Furthermore, capabilities are needed to enable hepatocyte model components to customize their responses, given a compound's molecular descriptors and/or physicochemical properties (PCPs). A method for predicting a new compound's CL is to discover a predictive correlation between patterns in large CL data sets and patterns in those compound's PCPs (Paixão et al., 2010). An operating hypothesis herein is that, long-term, the following, fundamentally different, four-part process will be easier and more intuitive, productive, and reliable. 1) Conceptually deconstruct hepatocyte cultures into abstract components and mechanisms, which can be instantiated as agents or objects in silico (via computer simulation). 2) Use only the more influential PCPs to parameterize specific drug-component events. 3) Assemble the component mechanisms in "hepatocytes" to mimic hepatocytes in culture. Execute and measure in silico clearance (CL_{is}), and 4) then use it to predict CL.

To explore feasibility, we elaborated the above four-part process into eight stages, four implementation and four experimentation. 1) Use object-oriented software tools to build an analog of hepatocytes in culture that actually exists (is not conceptual) to which objects representing drugs can be added. Call it an in silico hepatocyte culture (ISHC). 2) Mobile drug objects carry PCP information about a specific, referent compound. 3) Provide hepatocytes with biomimetic components to uptake, bind, and "metabolize" drug objects. Be parsimonious: avoid unneeded details. In silico mechanisms during sim-

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ABBREVIATIONS: CL, intrinsic clearance; PCPs, descriptors and/or physicochemical properties; CL_{is}, in silico clearance; ISHC, in silico hepatocyte culture; pCL_{is}, ISHC-predicted, in vitro intrinsic clearance; pCL_{MR}, clearance prediction from the multiple linear regression method; SM, similarity measure; IR, iterative refinement; BCS, biopharmaceutics classification system; P450, cytochrome P450; P-gp, P-glycoprotein; AUC, area under the concentration-time curve; AUFC, area under the fraction (of dose remaining) curve.

ulations will stand as compound-specific, explanatory hypotheses about how a CL profile is generated. Having explanatory mechanistic models necessarily precedes having predictive mechanistic models (Darden, 2007; Hunt et al., 2009). 4) Give hepatocyte components rules that specify using a small subset of PCPs to adjust their interactions with drug objects. 5) Provide a framework for conducting simulation experiments that mimic in vitro experiments. Measure compound-specific CL_{is} values. 6) Iteratively refine ISHCs so that CL_{is} values map to already-measured CL values. 7) Given a new compound's PCPs, use the preceding process to predict a CL value for that compound. 8) Use a protocol to iteratively revise and refine mappings from ISHC mechanistic details during simulations to in vitro counterparts, thereby enabling improved predictions.

With each cycle of iterative refinement, ISHCs are expected to become an incrementally improved, knowledge embodiment: our current best hypothesis about how PCPs and CL relationships are built into ISHC mechanisms. Prediction of in vivo hepatic clearance in humans will require additional, enhanced in silico capabilities. We envision plugging hepatocytes into validated in silico liver analogs (Park et al., 2009, 2010) within larger patient analogs using methods of the type described in Hunt et al. (2009) and Hunt and Ropella (2011). For the reasons presented in Hunt et al. (2008, 2009), the process can be synergistic with but is fundamentally different from methods used in what has been called model-based drug development (Rostami-Hodjegan and Tucker, 2007; Van Der Graaf and Gabrielsson, 2009; Allerheiligen, 2010; Suryawanshi et al., 2010). However, object- and agent-oriented methods are not alternatives to those conventional methods. Model uses are different. When combined, they can accomplish more than either approach alone (Hunt and Ropella, 2011).

An objective for this project was to challenge the above, eight-stage process by comparing clearance predictions based on ISHC experiments (pCL_{is}) with those obtained using a multiple linear regression method (pCL_{MR}). We set two targets. One was a similarity measure (SM): for 39 of 73 compounds for which multiple, literature-reported CL values were available, at least 50% of pCL_{is} values are ± 2 S.D. of the reported, mean CL values. A second measured relative performance: for all compounds, pCL_{is} performs at least as well as pCL_{MR}. Our iterative refinement protocol included selecting a small subset of PCPs, described as being important for each micromechanism, and then proposing a quantitative mapping from PCP space to ISHC mechanism space to parameterize each event in the absence of parameter tuning. We then conducted in silico clearance experiments, measured CL_{is} for each compound, and scaled those values to obtain pCL_{is}. The percentages of pCL_{is} and pCL_{MR} values that achieved SM targets for all 73 compounds were similar. For the subset of 39 cited above, pCL_{is} performed best. Thus, feasibility was demonstrated: the concrete detail built into ISHCs enabled acceptable performance. Improvement is needed before pCL_{is} values can be used with current methods to predict clearance in humans. We believe the approach presented has considerable potential for leveraging knowledge to predict in vitro metabolic clearance and eventually in vivo clearance, given only a new compound's PCPs and validated, explanatory, mechanistic insight instantiated in models such as ISHCs.

Materials and Methods

Hereafter, when describing ISHC features and components, we use SMALL CAPS to avoid confusion and make clear that ISHCs are not the same as the hepatocyte culture counterparts to which they map.

A goal was to design and construct an ISHC that would be no more complicated than needed to achieve stated uses. We cannot yet build accurate, precise, validated, predictive models of hepatocytes clearing xenobiotics. The reasons are that detailed cell and molecular biology knowledge is still insufficient and uncertainties are too great to do so. As explained in Hunt et al. (2009), the concept on which the following ISHC experiments are based is as follows. If the relative ISHC clearance properties of *m* COMPOUNDS are acceptably similar to their already measured in vitro values and key ISHC events map logically to hepatocyte counterparts, then a concretizable, quantitative map may exist between ISHC's PCP-based mechanistic details during CLEARANCE experiments and those occurring within hepatocytes. At that stage, given only the PCPs of a new compound, we can conduct ISHC experiments and obtain a CL_{is} value that can be transformed into a prediction of in vitro intrinsic clearance, pCL_{is}. The expectation is that the process and the pCL_{is} values can be improved iteratively.

During the sensitivity analysis described under *Results*, pseudo-random (simply random hereafter) sampling of the parameter space of the ISHC described below (Fig. 1) produced a set of nonidentical parameter vectors, which generated normalized CL_{is} values that were precise matches to each of the 39 different, normalized, mean CL values. From that result, we speculated that no additional detail would be needed to demonstrate (or not) feasibility. The challenge was whether we could discover at least one PCP-based, quantitative protocol to specify ISHC parameterizations so that similarity targets could be met. A future challenge will be to discover and improve additional protocols and develop methods to select the better performers.

Iterative Refinement Protocol. ISHC experiments must follow protocols, analogous to wet laboratory experiments. We followed the iterative refinement (IR) protocol in Fig. 2: cycles of ISHC assembly, testing and evaluation, validation or falsification, assessment, cogitation, and system revision. The process continues until a prespecified SM is satisfied. SMs typically begin weak and then are strengthened, as done by Lam and Hunt (2010). We have used the protocol successfully for different model types (Yan et al., 2008; Lam and Hunt, 2010; Tang and Hun, 2010; Engelberg et al., 2011). For this work, the primary attribute targeted in step 1 was that pCL_{is} mimics CL for a set of different compounds. INTRAHEPATOCYTE granularity is that illustrated in Fig. 1.

During each IR protocol cycle, mechanisms should be sufficiently complicated to achieve that cycle's goals, but no more so. Because an ISHC is an extensible, modular device, we know that we can add additional detail when needed and that doing so will be relatively straightforward. The parsimony guideline encourages resisting making an ISHC any more complicated than needed to achieve current and already achieved similarity targets, while leaving unspecified the many other mechanisms that might influence CL. We keep ISHC components simple by conflating fine-grained molecular biology details for which we have not yet demonstrated a need and representing them collectively using abstract objects and/or agents having relatively simple operating rules. There is a strong impulse to add mechanistic details (multiple metabolic pathways, for example) before their need has been demonstrated, simply because we have knowledge of those details and evidence that they may

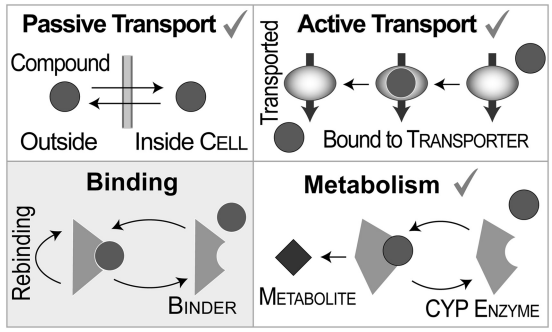

FIG. 1. Schematic diagram of the four ISHC micromechanisms. All events are parameter-specified probabilities. Checkmarks indicate that these events were made compound-specific based on PCPs. As stated under *Materials and Methods*, for this report, nonspecific binding was held constant. Passive transport: a COMPOUND can enter or exit a CELL passively. Active transport: an uptake or efflux TRANSPORTER binds a free COMPOUND; it remains attached for two time steps and is then released to the other side of the MEMBRANE. Nonspecific Binding: a BINDER binds a free COMPOUND; it is released after five time steps. Metabolism: ENZYMES are specialized BINDERS. They can bind and release a COMPOUND with probabilities $pEnzymeBind_i$ and $pRelease_i$; alternatively, rather than being released, the COMPOUND can be METABOLIZED.

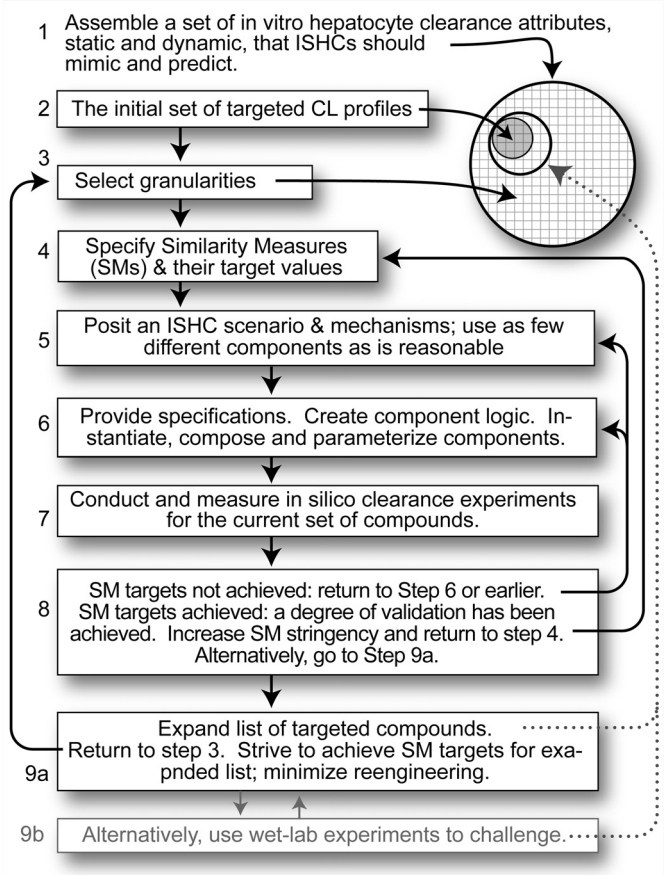

FIG. 2. An iterative refinement protocol for systematically developing and improving an ISHC.

contribute to CL. Doing so too early is a mistake for two reasons. 1) As explained in Hunt et al. (2009), we are not trying to build an accurate, detailed model of hepatocytes in cultures. 2) It can lead to inscription error, which is the logical fallacy of assuming the conclusion and programming in (consciously or otherwise) aspects of the result you expect to see. Once a degree of validation has been achieved, the behaviors of current components during simulation can be used for cross-model validation during refinement to counterparts having greater mechanistic detail.

When cycling through the IR protocol at some prespecified granularity (level of detail), three spaces are sampled and explored: mechanism (types of spaces, objects, and their arrangements), parameter (including the mapping from simulation cycles to wet laboratory time), and phenotype (the ISHC's behavior space). For this project we focused on just one phenotypic attribute, CL_{is}, but as demonstrated in other projects (Lam and Hunt, 2010; Park et al., 2010; Tang and Hunt, 2010; Engelberg et al., 2011), the focus can be a set of phenotypic attributes. For a complicated phenomenon such as CL, the reverse mapping from phenomenon to plausible generators is one-to-many (Hunt et al., 2009); many equally plausible mechanisms (networked events) can generate any one clearance profile. When dealing with hepatocytes, the mapping will be one-to-many no matter how much data we have, in part because there are differences in hepatocytes from the same liver; in addition, hepatocytes from the same source will be different at different times during the same study and/or at the same time under even slightly different culture conditions and so on. Given ample resources, the ideal scientific strategy (Hunt et al., 2009) would be to sample a variety of mechanisms and let them compete for survival through many IR protocol cycles. As discussed in Lam and Hunt (2010), we can shrink the space of mechanisms that may validate (for some prespecified granularity) by increasing the diversity of attributes targeted. As automated methods become available, it will become easier to explore multiple competing mechanisms in parallel.

For a specific mechanism, such as that in Fig. 1, as described under *Sensitivity Analysis*, only portions of the available parameter space can achieve validation targets, in part because we are striving to be parsimonious. However, because parameter influences are all networked during execution and the variability in outcomes between simulations can be nontrivial, there are many parameter vectors within that space that can produce essentially the same measured CL_{is}. However, as with mechanisms, when the number of attributes targeted is increased, the size of the parameter space that enables validation for that mechanism will shrink, sometimes to zero (Lam and Hunt, 2010). In the latter case, the mechanism is falsified. However, because of the design of ISHCs, revision is easy.

In this study, we are not exploring ISHC phenotype, the analog's behavior space. However, as part of a future project, we can do so. Two examples, unrelated to sensitivity analyses, follow. If data were available on the interaction of compounds A and B during CL studies, we could conduct in silico experiments to test the hypothesis that no A-B interaction occurs. If that is confirmed, the ISHC mechanism is falsified. If data were available on biliary excretion of compound C when hepatocytes are configured in sandwich cultures, as described in Sheikh-Bahaei et al. (2006), we could assemble them in silico as a sandwich culture and measure both CL_{is} and biliary excretion. If the latter is not similar to wet laboratory data, then the ISHC is falsified. We then revise the mechanism hypothesis until validation targets are achieved.

Model Components. We rely on object- and agent-oriented, discrete event software engineering methods (Zeigler, 1984; Zeigler et al., 2000; Hunt et al., 2009) coupled with relational grounding (discussed below). The basic methods have been described in Lam and Hunt (2009, 2010) and Sheikh-Bahaei et al. (2010). For this project, we reused the simple framework detailed in Sheikh-Bahaei and Hunt (2006) and Sheikh-Bahaei et al. (2006). Instructions for conducting in silico ISHC experiments and a description of the software are provided as supplemental data. ISHC refinement will be an ongoing process. The ISHC described herein is a snapshot of an early step in that process.

The essential components are an experiment agent, a CULTURE space, HEPATOCYTES, COMPOUNDS, and micromechanisms that enable a HEPATOCYTE to take up, bind, and METABOLIZE (or not) a COMPOUND. Additional components [e.g., subcellular features, as in Lam and Hunt (2010)] can be added easily when needed. Experiment agent follows a programmed protocol for managing the resources required for each experiment, controlling ISHCs, acquiring and recording telemetric data, progressing from one experimental setup to the next, and scoring simulation results against some performance measure. CULTURE space is a simple two-dimensional, 100 × 100 square grid. It maps to the culture media used. Because culture media does not typically change during an in vitro experiment, no culture media details were needed. A HEPATOCYTE is a composite agent that occupies one grid location. A composite agent is a container for other objects and agents, in this case, those in Fig. 1. All HEPATOCYTES are the same initially; 1000 were used. They map to a generalized representation of all human hepatocytes used in the cited studies. It is easy to individualize HEPATOCYTES to map to a specific study when the need arises. All HEPATOCYTES (all CELLS) are quasi-autonomous. A quasi-autonomous component simulates two of the following three properties; either stochastically or with a hidden determined algorithm, it is able to 1) choose its own response to stimulus, 2) act without stimulus, and 3) respond to a (new) stimulus, such as a new compound or context. In the latter case, it may misinterpret or mistranslate that stimulus. HEPATOCYTES possess properties 1 and 2; an objective of this work is to also give each the ability to satisfy property 3.

A COMPOUND is an independent, mobile object. To simulate the in vitro mixing that occurs during the several minutes (discussed below) to which a time step maps, each COMPOUND in the culture space was relocated randomly at the start of each time step. Thereafter, it could interact with HEPATOCYTES; 8000 COMPOUND objects (the DOSE) were randomly assigned to vacant grid locations at the start of each experiment. One compound maps to the amount of referent compound in a small aliquot of culture media. Other mappings have been used (Yan et al., 2008; Lam and Hunt, 2010; Park et al., 2010; Sheikh-Bahaei et al., 2010). During execution, individual event histories, such as a COMPOUND being transported out of a CELL or having moved into a CELL, can be tracked. Each COMPOUND carries identification information: a prespecified list of PCPs of its referent. Time advances discretely in simulation cycles (also called time steps). One time step maps to several minutes; the exact number depends on other quantitative mappings. During each time step, every component, selected in random order, is given an opportunity to update its status. It does so on the basis of local conditions at that instant, even though those

conditions may be different earlier and later in the simulation cycle. Random updating within simulation cycles simulates the parallel nature of events within cell cultures. Simulation results are recorded at the end of each simulation cycle. Those data map to snapshots of referent system details taken at regular intervals. No assumptions are made about events occurring between time steps.

The four micromechanisms in Fig. 1 are necessary to mimic metabolic clearance and proved sufficient to achieve objectives. When needed, those micromechanisms can be made more varied and/or complicated [more fine-grained, as done in Lam and Hunt (2010)], and additional micromechanisms can be added, as done in Lam and Hunt (2010), Sheikh-Bahaei et al. (2010), and Tang and Hunt (2010). Neither was required.

Each CELL agent is a container having a well mixed interior of unspecified capacity. The CELL controls passive compound movement in and out. There is no object that maps to the cell membrane because it was not needed: movement through a cell membrane is fast compared with a time step. Consequently, it is below an ISHC's spatial and temporal resolution. Each CELL detects all COMPOUNDS at adjacent, Moore neighborhood grid points at the start of each time step. A detected, free COMPOUND is given an opportunity to enter the CELL passively. The parameters *pCrossIn* and *CellCap* determine whether it enters. The former is the probability of entering a CELL passively within a time step. The latter places an upper limit on the amount of COMPOUND within a CELL during a time step; its value was fixed at 10 for all COMPOUNDS. Future ISHCs may map this parameter to solubility and/or biopharmaceutics classification system (BCS) class values (Wu and Benet, 2005). Each unbound COMPOUND within a CELL may also exit the CELL. The probability of doing so is controlled by *pCrossOut*. For simplicity $pCrossOut = pCrossIn/k$, where for this report $k = 100$. The reason for that choice is provided below. To determine whether a *CrossIn* event (or the event associated with any other probabilistic parameter) executes during a simulation cycle, the value of *pCrossIn* is compared with a random draw from a uniform 0–1 distribution; if the value drawn is less than *pCrossIn*, the event occurs.

There are two TRANSPORTER types per cell: uptake and efflux. A TRANSPORTER maps to a conflation of all components involved in active uptake or efflux mechanisms. We conjectured that, because active biliary excretion of compounds can be an important contributor to CL, the relative role of efflux transporters should be represented as being greater than that of uptake transporters. The current software (see supplemental data) enables implementation of biliary excretion when the referent is hepatocyte sandwich cultures (Sheikh-Bahaei and Hunt, 2006; Sheikh-Bahaei et al., 2006). For simplicity, one uptake and two efflux TRANSPORTERS were assigned to each CELL. A detected, external COMPOUND may be bound by an uptake TRANSPORTER with probability *pUptakeTransport* and then released into the CELL after two time steps. A free INTRACELLULAR COMPOUND may be bound by an efflux TRANSPORTER with probability *pEffluxTransport* and then released outside after two time steps. The choice of two time steps is somewhat arbitrary but is based on our earlier work (Sheikh-Bahaei and Hunt, 2006; Sheikh-Bahaei et al., 2006).

A nonspecific BINDER maps to a conflation of everything that can bind or sequester a compound. There are two types: external (to a CELL) and internal. The former maps to a conflation of everything on or in a cell membrane (or in the media) that can bind or sequester compounds. One each is assigned per CELL. For these ISHCs a BINDER can bind only one COMPOUND. When binding is made COMPOUND-specific, binding limitations can be changed so that there may or may not be a binding capacity limitation. Although only one COMPOUND was studied per experiment, it is easy to configure a binder to accommodate several different COMPOUNDS simultaneously. A detected COMPOUND may be bound to the external BINDER with probability *pNonSpecOutsideBind* and then released back to culture space after five time steps. The choice of five time steps is also somewhat arbitrary but is based on our earlier work (Sheikh-Bahaei and Hunt, 2006; Sheikh-Bahaei et al., 2006). A free INTRACELLULAR COMPOUND may be bound to the internal BINDER with probability *pNonSpecInsideBind* and then released within the cell after five time steps. Five time steps was selected somewhat arbitrarily because within a portion of a cell, compounds undergo many bind and release events within a few minutes. At both the beginning and end of that interval, a compound may be bound. Duration of binding can be made compound-specific during future rounds of ISHC refinement. We found no guidelines for relating nonspecific binding to PCPs. So, for this report, the values of *pNonSpecOutsideBind* and *pNonSpecInsideBind* were fixed at 0.1 for all compounds. We anticipate that in future ISHC variants, values of these parameters will be selected randomly from a range at the beginning of an in silico experiment. Future ISHC methods will need to include guidelines for doing so.

An ENZYME is similar to a BINDER. There are three ENZYMES per CELL. In Lam and Hunt (2010), one proved inadequate because, on occasion, it led to saturation artifacts. Having three proved adequate and matched TRANSPORTER number, so no more were used. Together, they map to a conflation of all enzymes that can metabolize a compound. Several of the 73 compounds are primarily metabolized by cytochrome P450 (P450) isozymes. Others, such as lorazepam and oxazepam, are glucuronidated. The abstract metabolism mechanism in Fig. 1 makes no assumptions about the chemical nature of a metabolite. Furthermore, at the current low level of resolution of ISHCs, we can start with a simplistic hypothesis that the influential PCPs will be similar for all metabolite classes. We elected to postpone metabolic specialization within ISHCs and explore that in future versions. Because there is considerable knowledge on how PCPs for a METABOLIC event influence interactions with P450 isozymes, for this project we elected to apply guidelines for P450-compound interactions with the expectation that a similar approach could be used for subclassification in future ISHC variants.

A free INTRACELLULAR COMPOUND may be bound to an enzyme with probability *pEnzymeBind*. It is held for two time steps and then either released unchanged with probability *pRelease* or METABOLIZED with probability *pMetabolize* ($pRelease = 1 - pMetabolize$). For simplicity, a metabolite is not created; the compound is simply destroyed.

Software. The ISHC code and instructions are available from the corresponding author. ISHCs were implemented using MATLAB. Marvin 4.1.7 (ChemAxon, Budapest, Hungary; 2007, http://www.chemaxon.com) calculator plug-ins were used for structure property prediction and calculation of molecular weight, rotatable bond count, log of the partition coefficient, log of the distribution coefficient, hydrogen bond donor count, pK_a, and polar van der Waals' surface area. Ionization potential and dipole moment were calculated using MOPAC 2007 (http://openmopac.net/).

Parameter Estimation. For a given set of compounds, $\{X_i | \ i = 1, \ldots, n\}$, the crucial task (stage 4, Introduction) was to devise relatively simple protocols for using PCPs to specify the parameter values of each event in Fig. 1. The feasibility of doing so was suggested by the success of "the rule of fours" method in predicting of P-glycoprotein (P-gp) substrate specificity (Didziapetris et al., 2003). Because all micromechanistic events were probabilistic, the value of each parameter was within the 0 to 1 range. After applying the above constraints, we were left with only six parameters that needed to be COMPOUND-specific: *pEnzymeBind*, *pEffluxTransport, pUptakeTransport, pMetabolize, pRelease,* and *pCrossIn.* During ISHC execution, measures were taken of the fraction of initial COMPOUND dose remaining in the CULTURE external to CELLS. Those values were used to calculate CL_{is}.

Micromechanisms and Parameterization Guidelines. Are the micromechanisms in Fig. 1 too simplistic? Is the range of CL_{is} values at least as great as the range in CL values? The sensitivity analysis described under *Results* showed that the first answer is no and the second is yes. We assigned parameter values randomly and obtained results from hundreds of simulation experiments that had clearance properties characteristic of those of the wet laboratory clearance experiments. From those, we obtained the range of normalized CL_{is} values that can be achieved by using ISHCs. The *Z* score range (see below) for that set was greater than that for normalized CL values used in this study. For each of 73 normalized CL values, several normalized CL_{is} values matched, each having a different parameter vector and thus differently parameterized micromechanisms. Our operating hypothesis was that a set of PCP-based guidelines could be devised and used to parameterize ISHCs so that a significant fraction of CL_{is} values could be transformed, as described below, to pCL_{is} values that would be similar to corresponding CL values. The task was to develop a prototype set of guidelines, apply them, and compare the results with those obtained using a control or reference method. For the latter, we specified multiple linear regressions, discussed below. The reference method would be used to predict CL, given PCPs (pCL_{MR}). If results from the prototype guidelines were worse than those from the regression method, then we could conclude that we were on the wrong track. If they were similar or better, that would be encouraging and supportive of feasibility. We could then seek strategies to improve the prototype guidelines.

TABLE 1

Known influence of PCPs on compound interactions with P450 isozymes

An upward arrow indicates positive correlation, and a downward arrow indicates negative correlation.

PCP	CYP3A4 Binding	CYP2D6 Binding	CYP2C9 Binding	CYP1A2 Binding	CYP2C19 Binding	CYP2E1 Binding	Enzyme Binding	Rate of Metabolism
Size: MW	↓ [a]	↑ [b]			↑ [b]	↓ [a,b,c]		
Flexibility: RBC			↑ [a]	↑ [a]	↑ [a]		↑ [a]	
Lipophilicity								
logP	↑ [a,c]	↑ [c]	↑ [a,b,c]	↑ [c]	↑ [b]	↑ [c]		
logD							↑ [a]	
Acidity								
HBD			↑ [a,b,c]	↑ [b]	↓ [a,b]	↑ [b]		
HBA			↑ [a]	↑ [b]	↑ [a]	↑ [a]		
Ionization								
pK_a	↑ [c]		↑ [b]	↑ [c]				
MFI								↑ [a]
IP (HOMO)	↑ [b]			↓ [b]				↑ [a]
Polarity								
PSA		↑ [a]						
DM				↓ [b]				

MW, molecular weight; RBC, rotatable bond count; logP, log of the partition coefficient; logD, log of the distribution coefficient; HBD, hydrogen bond donor count; HBA, hydrogen bond acceptor count; MFI, maximum fraction ionized at pH = 7.4; IP, ionization potential; HOMO, highest occupied molecular orbit; PSA, polar van der Waals' surface area; DM, dipole moment.

[a] Lewis et al., 1998.

[b] Lewis et al., 2002.

[c] Smith et al., 1997.

We drew on information in three expert reviews (Smith et al., 1997; Lewis et al., 1998, 2002) to specify broadly how PCPs are believed to influence the in vitro counterpart of each event in Fig. 1. Table 1 shows how the cited literature describes specific PCPs influencing interactions with P450s. Table 2 shows how specific PCPs influence features of cell permeability. From this information, we sought rough, yet particular, heuristic guidelines for how relative differences in PCPs might be expected to influence the probability of a specific ISHC event. Any number of such guidelines can be stated. At this stage, we were not seeking those that worked best, just one set that was reasonably effective and consistent with current knowledge. For example, when ENZYME maps primarily to CYP2C9, then *pEnzymeBind* (a probability) should be larger for a COMPOUND assigned a larger logP; in addition, a COMPOUND assigned a larger ionization potential should have a larger *pMetabolize*. From these guidelines and others, we specified a parameter influence score (S) for each Fig. 1 event: $S_{event} = (w_1 \cdot PCP_{1_z} + w_2 \cdot PCP_{2_z} + \ldots + w_n \cdot PCP_{n_z})/c$. Subscript z indicates that all values have been transformed using a standard Z score method. Standard normal distribution allows negative values, but some PCPs cannot have negative values. In future ISHCs different distributions will need to be considered, for example, lognormal and Poisson, and their respective transformations. w_j is a scalar; it determines the degree to which PCP_j influences S. The probabilistic parameter value for an event that has the largest S should be near or equal to 1.0, and the parameter value for the compound that has the smallest S should be near 0. When w_j is positive (negative) PCP_j is positively (negatively) correlated with S. We conjectured that the PCPs more frequently identified in our citations as being influential should be given a greater weight. In the absence of any guidelines for specifying w values, a parsimonious rule was to simply give each w a value of 1, 2, or 3, corresponding to the number of Table 1 references that described that particular influence. It is clear that as ISHC methods are improved, knowledge-based guidelines for specifying w can be developed. The sum of the weighted, transformed PCPs was divided by $c = 3$ because that is the maximum number of citations.

TABLE 2

Known influence of PCPs on compound interactions with cells, membranes, and transporters

An upward arrow indicates positive correlation, and a downward arrow indicates negative correlation.

PCP	Membrane Permeability	Caco-2 Permeability	P-gp Substrate[a]	P-gp Nonsubstrate[a]
MW	↓ [b]		>400[c]	<400[c]
ONC			≥8[c]	≤4[c]
$logD_{7.4}$		↑ [d]		
logP	↑ [b]			
Acidic pK_a			<4[c]	
Basic pK_a				<8[c]
IP	↓ [b]			
PSA	↑ [b]	↓ [d]		

MW, molecular weight; ONC, O + N atom count; logP, log of the partition coefficient; $logD_{7.4}$, log of the distribution coefficient at pH = 7.4; IP, ionization potential; PSA, polar van der Waals' surface area.

[a] Rule of fours (Didziapetris et al., 2003).

[b] McGinnity et al., 2004.

[c] Didziapetris et al., 2003.

[d] Artursson et al., 2001.

The following 12 normalized influence score rules were constructed using the information in Tables 1 and 2. The abbreviations are provided in Tables 1 and 2. It is clear that in each case, many other options can be suggested that may work as well or better. A strategy for dealing with those options is provided under *Discussion*. Normalized score values were then transformed into the required, probabilistic ISHC parameter values.

1. CYP3A4 binding score: $BS_{3A4} = (-MW_z + 2 \cdot logP_z + IP_z + pK_{a,\,acidic,\,z})/3$
2. CYP2D6 binding score: $BS_{2D6} = (MW_z + logP_z + PSA_z + DM_z)/3$
3. CYP2C9 binding score: $BS_{2C9} = (RBC_z + 3 \cdot logP_z + 3 \cdot HBD_z + HBA_z - pK_{a,\,acidic,\,z})/3$
4. CYP1A2 binding score: $BS_{1A2} = (RBC_z + logP_z + HBD_z + HBA_z + pK_{a,\,acidic,\,c} - IP_z - DM_z)/3$
5. CYP2C19 binding score: $BS_{2C19} = (MW_z + RBC_z + logP_z + HBA_z - HBD_z)/3$
6. CYP2E1 binding score: $BS_{2E1} = (-MW_z + 2 \cdot logP_z + HBD_z + HBA_z)/3$
7. Enzyme binding score: $BS_{Enz} = (RBC_z + logD_{7.4,\,z})/3$
8. P450 rate of metabolism score: $MS_{p450} = MFI_z + IP_z$
9. P-gp substrate score: $SS_{P\text{-}gp} = (MW - 400)/400 + (ONC - 8)/8 + (4 - pK_{a,\,acidic,\,z})/4$
10. P-gp nonsubstrate score: $NS_{P\text{-}gp} = (400 - MW)/400 + (4 - ONC)/4 + (8 - pK_{a,\,basic,\,z})/8$
11. MEMBRANE permeability score: $MPS = -MFI_z + PSA_z + logP_z - MW_z$
12. Caco-2 permeability score: $C2PS = logD_{7.4,\,z} + PSA_z$

The P-gp substrate and nonsubstrate scores were based on the rule of fours method for predicting P-gp substrate specificity (Didziapetris et al., 2003). We considered only the above six major P450 isozymes because they are primarily responsible for the metabolism of many xenobiotics in man (Smith et al., 1997). Their percentage involvement is 34% for CYP3A4, 19% for CYP2D6, 16% for CYP2C9, 8% for CYP1A2, 8% for CYP2C19, and 4% for CYP2E1 (Lewis et al., 2002). Because enzyme conflates all metabolizing enzymes, an

overall binding score BS) was needed. For simplicity, we conjecture that for this work the P450 rules could serve as placeholders for corresponding rules for other metabolizing enzymes. We used the following, weighted, linear sum of the individual binding scores. The sum of the involvement of the six isozymes is 89%; we therefore included the overall metabolizing enzyme binding score and gave it a weight of 0.11: $BS = 0.34 \cdot BS_{3A4} + 0.19 \cdot BS_{2D6} + 0.16 \cdot BS_{2C9} + 0.08 \cdot BS_{1A2} + 0.08 \cdot BS_{2C19} + 0.04 \cdot BS_{2E1} + 0.11 \cdot BS_{Enz}$.

There are any number of ways to construct a BS. We sought just one. Future ISHC variants will need to explore others. The next task was to quantitatively map a BS onto a probability interval [minimum, maximum] to obtain an ISHC parameter value. In all but one case, we used [0.05, 0.95] rather than [0, 1.0] to reserve the most extreme probability values for future compounds. As the number of compounds in the set increases, the likelihood increases that one or more compounds will have a *pEnzymeBind* outside the current range (Supplemental Table S1). We conjectured that for the compound having the maxBS, the probability of encountering and binding to an enzyme within a simulation cycle (*pEnzymeBind*) should be close to 1.0. We arbitrarily set the upper limit to 0.95. As the number of compounds under study increases, a protocol can be developed for setting upper and lower limits. If we were to increase the number of enzymes within each cell, we could proportionally decrease the upper limit value. With the exception of *pCrossIn*, we did the same for the other events in Fig. 1. We specified that for the compound having minBS, *pEnzymeBind* should be >0. We arbitrarily specified that, within ISHCs, minBS = 0.05. With the exception of *pCrossIn*, we did the same for the other parameter values: $pEnzymeBind_i = 0.9(BS_i - \text{minBS})/(\text{maxBS} - \text{minBS}) + 0.05$.

The weight of expert opinion, based in part on energy barriers (McCammon and Harvey, 1987; Beveridge and DiCapua, 1989), is that the probability of remaining bound is greater than the probability of binding, i.e., $1 - pRelease \geq pEnzymeBind$. For simplicity, we specified that $1 - pRelease$ be a function of *pEnzymeBind*, specifically $1 \geq f(pEnzymeBind) \geq pEnzymeBind$ for all values of *pEnzymeBind*. There are many options for *f*(*pEnzymeBind*). We arbitrarily specified that $f(pEnzymeBind) = \sqrt{(pEnzymeBind)}$. Thus, within ISHCs: $pRelease_i = 1 - \sqrt{(pEnzymeBind_i)}$.

We used the same method to map the P450 rate of metabolism score (MS_{p450}) onto the [0.05, 0.95] probability interval to obtain a probability of METABOLISM value: $pMetabolize_i = 0.9(MS_i - \text{minMS})/(\text{maxMS} - \text{minMS}) + 0.05$.

The above list contains P-gp substrate and nonsubstrate scores. We combined them into one active efflux transport score using $TS = (SS_{P\text{-}gp} - NS_{P\text{-}gp})/2$ and then mapped that composite score onto the [0.05, 0.95] probability interval as above to obtain a per time step efflux transport probability value: $pEffluxTransport_i = 0.9(TS_i - \text{minTS})/(\text{maxTS} - \text{minTS}) + 0.05$.

We specified that the Caco-2 permeability score C2PS should approximate the sum of a cell's passive uptake transport score (UTS) and its active transport score, TS (C2PS ~ UTS + TS); thus, UTS = C2PS − TS. We used the same method to map UTS onto the [0.05, 0.95] interval to obtain uptake transport probability: $pUptakeTransport_i = 0.9\{UTS_i - \min(UTS)\}/\{\max(UTS) - \min(UTS)\} + 0.05$.

The fraction of a hepatocyte culture occupied by cells is typically very small, whereas CELLS occupy 10% of CULTURE space. Thus, as done in Sheikh-Bahaei and Hunt 2006; Sheikh-Bahaei et al., 2006), some exploratory simulations were needed to establish an upper limit for the probability of a COMPOUND entering a CELL within a simulation cycle. For membrane cross-in probability, we mapped MPS to [0.01, 0.51]. We specified that for the COMPOUND having min(MPS), *pCrossIn* should be >0. From the cited exploratory studies, we learned that setting min(MPS) = 0.01 was adequate: $pCrossIn = 0.51\{MPS_i - \min(MPS)\}/\{\max(MPS) - \min(MPS)\} + 0.01$ and $pCrossOut = 0.01 \cdot pCrossIn$.

The reason for 0.01 was to make cross-out and cross-in "rates" approximately the same for each COMPOUND under steady-state conditions in the absence of METABOLISM; we had no evidence that they should be different. A multiplier much less than 1.0 was needed in part because the grid space occupied by a CELL maps to a much smaller volume than does a CULTURE grid space. Furthermore, during each simulation cycle, only a small fraction of COMPOUNDS within CULTURE space have an opportunity to passively enter a CELL, whereas all unbound COMPOUNDS inside a CELL are given an opportunity to exit. Lam and Hunt (2010) handled the permeation events differently. In future ISHCs, the pros and cons of additional approaches need to be explored.

In Vitro Data, Similarity Measures, and Similarity Targets. Table 3 lists reported CL values of 73 compounds obtained using human hepatocyte cultures (Schneider et al., 1999; Lau et al., 2002; McGinnity et al., 2004). Of those, 39 had two or more independently measured CL values, enabling calculation of a S.D. and coefficient of variation. To demonstrate feasibility we needed to compare ISHC predictions, using quantitative metrics, with those from multiple linear regression (discussed below). We conjectured that to demonstrate feasibility, the ISHC approach would need to perform at least as well as multiple linear regression. We needed one or more measures of performance. The mean coefficient of variation for the subset of 39 was 0.75. Given that level of variation, we specified two SMs and three similarity targets. SM1: for the subset of 39 (having calculated S.D.s), pCL_{is} or pCL_{MR} is within CL ± 2 S.D. Target 1: ≥50% of pCL_{is} achieve SM1. Target 2: the percentage of pCL_{is} that achieve SM1 is at least twice that for pCL_{MR}. SM2: pCL_{is} or pCL_{MR} is within a factor of 2 of the referent CL. Target 3: the percentage of pCL_{is} that achieve SM2 is greater than or equal to the percentage of pCL_{MR} that do so.

Targets 1 to 3 are relatively weak. Why do we not strive to achieve more stringent SM targets? Experience in using the IR protocol has taught that striving prematurely toward stringent SM targets during early IR protocol cycles increases risks of downstream problems; see Lam and Hunt (2010) for examples. Adding and parameterizing plausible mechanistic details will enable achievement of a more stringent SM target, but those details (even though they may seem reasonable) have not survived falsification challenges. We know that during a subsequent IR protocol cycle addition of a new, targeted attribute will falsify the current ISHC and its mechanisms. When there are many, unvalidated mechanistic features, unraveling those mechanistic details to locate the source of failure (why it was falsified) can become tedious and may require extensive reengineering and revalidation. Starting with relatively weak SM targets that can be achieved with relatively simple mechanisms and parameterizations and then moving forward (additional IR protocol cycles) in small steps is more efficient because it is grounded to the scientific process.

Also listed in Table 3 is each compound's class according to the BCS (Wu and Benet, 2005): 17 in class 1 (high permeability, high solubility), 6 in classes 2 (high permeability, low solubility) and 3 (low permeability, high solubility), and 1 in class 4 (low permeability, low solubility). BCS classifications were not available for the other compounds, and we did not undertake classifying them.

Clearance Measurement and Prediction. In vitro intrinsic clearance can be calculated using area under the concentration-time curve (AUC) and dose: CL = dose/AUC. We used that method to calculate CL_{is}. The time course of fraction, *F*, of COMPOUNDS in the EXTRACELLULAR CULTURE space was recorded, and the area under that fraction curve, AUFC, was calculated. AUFC maps to AUC/dose. Consequently, $CL_{is} = 1/\text{AUFC}$. The long-term ISHC design goal is that there be a predictive, quantitative mapping from CL_{is} to CL values. The simplest relationship is linear, so we hypothesized that for a new *i*th compound, $pCL_{is,\,i} = s_i \cdot CL_{is}$, where s_i is a scaling factor and that the relationship will enable achievement of similarity targets 1 to 3. We searched for a value of *s* such that all three targets were achieved. Examples are available in Supplemental Table S2. We optimized s_i so that the number of COMPOUNDS achieving the similarity targets was maximized. $pCL_{is,\,i}$ values were calculated in a leave-one-out manner as follows: the *i*th value was taken out of the set of 39 CL_{is} values. An s_i value was chosen such that it optimally transformed the remaining 38 CL_{is} values within the ±2 S.D. range. In all cases, those values were close to 5. The PCPs of the 39th COMPOUND were used to estimate a set of simulation parameters. After 10 Monte Carlo simulations, its mean CL_{is} was calculated as described above and then scaled to give its pCL_{is} value. Ten Monte Carlo simulations were more than adequate because all S.D.s were small, <0.02. Once targets 1 to 3 were achieved for the subset of 39 compounds, we expanded attention to all 73 compounds and targets 1 to 3.

For comparison, intrinsic clearance values were predicted using a standard, multiple linear regression method. Details are provided in supplemental data. pCL_{MR} values were also calculated in a leave-one-out manner. Independent variables were the PCPs listed in Table 1.

Relational Grounding and ISHC-to-In Vitro Mapping. To improve ISHCs sufficiently so that they can be useful in predicting in vivo clearance, it will be necessary to cycle through the IR protocol many additional times, preferably using automated methods. Doing so with limited reengineering problems requires that ISHCs use relational grounding. How an ISHC or any other model is grounded affects how it can and should be used. The units,

TABLE 3

Comparisons of reported CL values with various computed clearance values

Compound	BCS Class[a]	CL[b]	*n*	S.D.	2-Fold Range for CL		CL_{is}	pCL_{is}[c]	Within 2-Fold Range	pCL_{MR}	Within 2-Fold Range	CL ± 2 S.D.[d]		pCL_{is}[e]	Within ±2 S.D.	pCL_{MR}	Within ±2 S.D.
		$\mu l \cdot min^{-1} \cdot 10^{6}\ cells^{-1}$															
Acebutolol	N.A.	1.8	4	1.5	0.9	3.6	0.20	0.87	N	7.6	N	−1.2	4.8	0.85	Y	5.4	N
Antipyrine	1	0.3	2	0.3	0.14	0.57	0.27	1.22	N	−2.4	N	−0.2	0.8	1.14	N	−2.7	N
Atenolol	3	0.5	5	0.5	0.25	1	0.17	0.77	Y	5.2	N	−0.5	1.5	0.72	Y	−17.8	N
Bepridil	N.A.	2.0	1		1	4	0.27	1.25	Y	14.2	N						
Betaxolol	N.A.	2.5	6	1.0	1.25	5	0.19	0.89	N	4.3	Y	0.5	4.5	0.83	Y	1.1	Y
Bisoprolol	N.A.	1.6	8	1.4	0.8	3.2	0.19	0.89	Y	7.5	N	−1.2	4.4	0.83	Y	5.6	N
Bosentan	N.A.	0.2	1		0.1	0.4	0.26	1.22	N	−18.3	N						
Bromocriptine	N.A.	37.0	1		18.5	74	0.24	1.10	N	8.5	N						
Caffeine	1	1.6	3	1.6	0.8	3.2	0.21	0.99	Y	22.4	N	−1.6	4.8	0.92	Y	12.6	N
Carbamazepine	2	2.0	1		1	4	0.27	1.22	Y	13.7	N						
Carvedilol	2	35.0	5	11.0	17.5	70	0.23	1.06	N	1.9	N	13.0	57.0	0.99	N	−1.3	N
Cetirizine	N.A.	0.5	5	0.5	0.25	1	0.15	0.70	Y	9.1	N	−0.5	1.5	0.65	Y	2.0	N
Chlorpheniramine	1	2.8	4	1.3	1.4	5.6	0.24	1.10	N	14.7	N	0.2	5.4	1.03	Y	21.8	N
Chlorpromazine	2	11.0	1		5.5	22	0.26	1.18	N	10.2	Y						
Chlorprothixene	N.A.	14.0	1		7	28	0.26	1.18	N	9.6	Y						
Cimetidine	3	1.2	9	0.4	0.6	2.4	0.25	1.14	Y	4.4	N	0.4	2.0	1.07	Y	−12.9	N
Clozapine	N.A.	6.0	1		3	12	0.12	0.56	N	3.1	Y						
Codeine	N.A.	23.0	2		11.5	46	0.09	0.40	N	11.1	N						
Cyclosporine	2	3.5	8	1.5	1.75	7	0.30	1.36	N	12.9	N	0.5	6.5	1.27	Y	28.0	N
Desipramine	1	7.0	2	5.7	3.5	14	0.19	0.88	N	15.6	N	−4.3	18.3	0.83	Y	25.6	N
Dextromethorphan	N.A.	7.6	81	8.1	3.8	15.2	0.26	1.19	N	10.1	Y	−8.6	23.8	1.11	Y	1.3	Y
Diazepam	1	0.8	3	0.6	0.4	1.6	0.28	1.28	Y	33.7	N	−0.3	1.9	1.20	Y	121.9	N
Diltiazem	1	8.9	90	5.0	4.44	17.75	0.20	0.91	N	13.0	Y	−1.2	18.9	0.85	Y	19.0	N
Diphenhydramine	1	6.0	1		3	12	0.25	1.15	N	12.5	N						
Doxepin	1	13.0	1		6.5	26	0.20	0.93	N	6.3	N						
Ethinylestradiol	1	7.0	5	2.0	3.5	14	0.29	1.33	N	2.4	N	3.0	11.0	1.24	N	3.8	Y
Famotidine	3	0.5	6	0.5	0.25	1	0.20	0.93	Y	2.2	N	−0.5	1.5	0.87	Y	−33.6	N
Felodipine	N.A.	7.5	1		3.75	15	0.29	1.35	N	9.8	Y						
Fluoxetine	1	1.0	1		0.5	2	0.25	1.13	Y	7.2	N						
Furosemide[f]	3,4	0.5	1		0.25	1	0.21	0.96	Y	2.4	N						
Granisetron	N.A.	9.0	4	8.7	4.5	18	0.17	0.79	N	5.7	Y	−8.4	26.4	0.74	Y	4.3	Y
Ibuprofen	2	4.2	1		2.1	8.4	0.23	1.06	N	9.3	N						
Imipramine	1	8.1	4	2.0	4.03	16.1	0.24	1.12	N	8.5	Y	4.0	12.1	1.04	N	6.0	Y
Isradipine	N.A.	18.0	1		9	36	0.29	1.34	N	34.8	Y						
Lorazepam	N.A.	0.5	3	0.4	0.26	1.05	0.30	1.36	N	20.6	N	−0.3	1.3	1.27	Y	39.2	N
Methylprednisolone	N.A.	9.7	1		4.85	19.4	0.28	1.29	N	10.0	Y						
Metoprolol	1	7.0	5	2.9	3.5	14	0.16	0.73	N	10.9	Y	1.2	12.8	0.69	N	16.3	N
Mibefradil	N.A.	0.9	1		0.45	1.8	0.23	1.04	Y	18.5	N						
Midazolam	1	11.6	7	7.7	5.82	23.29	0.28	1.29	N	−0.3	N	−3.8	27.1	1.20	Y	−9.9	N
Mofarotene	N.A.	2.0	1		1	4	0.31	1.44	Y	4.0	Y						
Morphine	N.A.	24.0	2		12	48	0.10	0.46	N	34.9	Y						
Nadolol	3	0.5	3	0.5	0.25	1	0.15	0.67	Y	18.2	N	−0.5	1.5	0.63	Y	19.7	N
Naloxone	N.A.	86.9	3	111.9	43.45	173.8	0.15	0.71	N	2.6	N	−137	310	0.66	Y	−36.1	Y
Nicardipine	N.A.	7.3	1		3.65	14.6	0.23	1.07	N	11.6	Y						
Nifedipine	1	6.0	6	1.6	2.98	11.9	0.29	1.31	N	7.5	Y	2.8	9.1	1.23	N	4.6	Y
Nilvadipine	N.A.	13.3	1		6.65	26.6	0.30	1.37	N	16.7	Y						
Nitrendipine	N.A.	8.0	8	3.6	3.99	15.95	0.30	1.38	N	12.0	Y	0.7	15.2	1.29	Y	25.3	N
Omeprazole	N.A.	1.7	2		0.85	3.4	0.22	1.03	Y	13.5	N						
Ondansetron	N.A.	1.4	5	0.5	0.7	2.8	0.21	0.98	Y	15.9	N	0.4	2.4	0.85	Y	−1.3	N
Oxazepam	N.A.	1.2	2	1.1	0.6	2.4	0.29	1.34	Y	25.5	N	−1.1	3.5	1.25	Y	39.3	N
Pindolol	N.A.	2.8	4	1.0	1.4	5.6	0.18	0.83	N	5.3	Y	0.8	4.8	0.77	N	3.5	Y
Pirenzepine	N.A.	0.5	4	0.5	0.25	1	0.08	0.35	Y	15.2	N	−0.5	1.5	0.33	Y	23.2	N
Prazosin	N.A.	2.3	7	1.7	1.15	4.6	0.20	0.90	N	19.6	N	−1.1	5.7	0.85	Y	36.4	N

TABLE 3—*Continued*

Compound	BCS Class[a]	CL[b]	*n*	S.D.	2-Fold Range for CL		CL_{is}	pCL_{is}[c]	Within 2-Fold Range	pCL_{MR}	Within 2-Fold Range	CL ± 2 S.D.[d]		pCL_{is}[e]	Within ±2 S.D.	pCL_{MR}	Within ±2 S.D.
Prednisolone	1	9.7	1		4.85	19.4	0.27	1.25	N	7.5	Y						
Propofol	N.A.	107.0	5	26.0	53.5	214	0.30	1.37	N	−1.2	N	55	159	1.28	N	−20.5	N
Propranolol	1	9.9	92	5.0	4.97	19.87	0.19	0.89	N	10.3	Y	0.0	19.9	0.83	Y	28.4	N
Ranitidine	3	1.0	5	0.1	0.5	2	0.23	1.04	Y	11.8	N	0.9	1.1	0.90	Y	9.0	N
Remikiren	N.A.	19.5	1		9.75	39	0.30	1.36	N	−8.0	N						
Ritonavir	2	2.1	7	3.0	1.05	4.2	0.31	1.42	Y	−1.3	N	−3.9	8.1	1.33	Y	17.9	N
Scopolamine	N.A.	7.0	1		3.5	14	0.19	0.86	N	5.7	Y						
Sildenafil	N.A.	5.2	1		2.6	10.4	0.23	1.06	N	14.8	N						
Sulpiride[f]	N.A.	0.5	1		0.25	1	0.13	0.62	Y	2.8	N						
Temazepam	N.A.	2.0	2		1	4	0.28	1.30	Y	−21.3	N						
Tenoxicam	N.A.	2.6	1		1.3	5.2	0.18	0.81	N	9.3	N						
Terbutaline[f]	N.A.	0.5	1		0.25	1	0.18	0.81	Y	−2.5	N						
Theophylline	1	0.3	2	0.3	0.16	0.62	0.15	0.69	N	13.2	N	−0.3	0.9	0.64	Y	59.0	N
Tolbutamide	N.A.	1.6	1		0.8	3.2	0.21	0.97	Y	−8.2	N						
Tolcapone	N.A.	1.2	1		0.6	2.4	0.27	1.26	Y	8.7	N						
Triazolam	N.A.	1.0	2		0.5	2	0.31	1.43	Y	2.8	N						
Triprolidine	N.A.	4.3	4	3.3	2.15	8.6	0.25	1.17	N	10.9	N	−2.3	10.9	1.10	Y	32.3	N
Verapamil	1	18.0	87	11.9	8.99	35.95	0.30	1.37	N	10.6	Y	−5.9	41.8	1.28	Y	11.2	Y
Warfarin	2	1.1	1		0.55	2.2	0.25	1.15	Y	12.0	N						
Zileuton	N.A.	2.1	16	1.8	1.05	4.2	0.24	1.10	Y	−3.6	N	−1.5	5.7	1.03	Y	−15.5	N
									28 of 73 (38%)		23 (32%)				31 of 39 (79%)		9 (23%)

N.A., not applicable; N, no; Y, yes.

[a] BCS class: 1, high permeability, high solubility; 2, high permeability, low solubility; 3, low permeability, high solubility; 4, low permeability, low solubility.

[b] Human, in vitro CL compiled from Lau et al., 2002, McGinnity et al., 2004, and Schneider et al., 1999.

[c] Scale values by which each CL_{is} value is multiplied. Default values are $\min(s_i)$, where s_i is the optimal scale factor found by the leave-one-out method; for the 2-fold range, $s_i = 4.6$. See Supplemental Table S2 for details and additional ranges. pCL_{is} has the same units as CL.

[d] Comparisons are made only for the 39 compounds for which two or more CL values were available.

[e] For the 39 CL values for which S.D. values were available, $s_i = 4.3$; 64% of pCL_{is} and 21% pCL_{MR} were within the ±1.5 S.D. range. See Supplemental Table S2 for additional ranges.

[f] 0.5 was used for undetectable CL values (reported as being ≤1).

dimensions, and/or objects to which a variable or model constituent refers establish groundings. Inductive models, such as pharmacokinetic and classic compartment-based clearance models, are typically grounded absolutely. So doing provides simple, interpretive mappings between output and parameter values and referent data. Absolute grounding creates issues that must be addressed each time one needs to expand the model to include additional phenomena and when one combines models to form a larger system. Addition of a term to an equation, for example, requires defining its variables and premises to be quantitatively commensurate with everything else in the model. Such expansions can be challenging and even infeasible when there are multiple uncertainties, and knowledge is limited. A model composed of components (or terms) all grounded to the same metric spaces, such as a pharmacokinetic model, for example, is itself grounded to the Cartesian composite of all those metric spaces. The reusability of such a model is limited under different experimental conditions or when an assumption made is brought into question. ISHC components and processes are grounded to each other; they need not have assigned units. Such relational grounding enables synthesizing flexible, easily adaptable models. However, a separate mapping model (e.g., $pCL_{is,\,i} = s_i \cdot CL_{is}$) that provides the required units is needed. See Hunt et al. (2009) for a more detailed discussion of the merits and limitations of relational grounding.

Results

Sensitivity Analysis. We created a set of 1000 ISHCs, each having unique, randomly assigned values of *pEnzymeBind*, *pEffluxTransport*, *pUptakeTransport*, *pMetabolize*, *pRelease*, and *pCrossIn*. For each ISHC, we ran CLEARANCE experiments and plotted log(EXTRACELLULAR fraction of DOSE) versus TIME. Those CL_{is} profiles were categorized into two groups on the basis of curve shape: acceptable and unacceptable. A CL_{is} profile was categorized as acceptable if it was log-linear. There were five subtypes of unacceptable profiles: biexponential, abiotic, saturated (after an interval, CLEARANCE ceased), bumpy biexponential, and bumpy log-linear. Examples from each category along with details of the sensitivity analysis are provided in the supplemental data and Supplemental Figs. S5 to S13. Clear trends were seen. There were regions of parameter space in which a particular type was more numerous. For example, in a plot of *pUptakeTransport* versus *pMetabolize*, most of the biexponential CL_{is} profiles were within the subspace where both parameter values were <0.5. Additional examples are provided as supplemental figures.

Log-linear CLEARANCE profiles exist in all regions, but they were more numerous in the following parameter subspace: *pMetabolize* $>$ 0.5, *pEnzymeBind* $>$ 0.5, and *pUptakeTransport* $<$ 0.5. Values of CL_{is} were calculated for all log-linear profiles. The correlation was very weak between $logCL_{is}$ and values of each individual parameter, indicating that a particular CLEARANCE profile is somewhat robust to changes in any one parameter. As might be expected, the two largest correlation coefficients were for *pEnzymeBind* ($r^2 = 0.19$) and *pMetabolize* ($r^2 = 0.06$). However, because all events are networked, small changes (e.g., 0.1) in all parameters can cause easily measured changes in clearance, and, on occasion, can change a clearance profile from acceptable to unacceptable.

Prediction Performances. The ISHC parameter vector for each compound is listed in Supplemental Table S1. Listed in Table 3 for each compound are the average CL_{is} values (10 Monte Carlo experiments), the SM1 and SM2 target ranges, the ISHC predicted in vitro clearance values, pCL_{is}, and the in vitro clearance values predicted from multiple linear regression, pCL_{MR}. Likewise, targets 1 and 2 were achieved: 79% of pCL_{is} values achieved SM1 (for the subset of 39, the predicted value is within CL $\pm$ 2 S.D.), whereas 23% of pCL_{MR} achieved SM1. The performances of both pCL_{is} and pCL_{MR} were poorer when comparisons were extended to all 73 compounds. Nevertheless, similarity target 3 was achieved: the percentage of pCL_{is} that achieved SM2 (predicted value within a factor of 2 of referent CL) was greater than the percentage of pCL_{MR} that did so. To be specific, 38% of pCL_{is} and 32% of pCL_{MR} achieved SM2. Taken together, those results support feasibility yet indicate that considerable refinement is needed to make ISHC predictions useful for supporting research and development decision making. When we limit attention to pCL_{is} values for the individual BCS classes within the subset of 39, we see that 8 of 13 (62%) class 1 COMPOUNDS, 2 of 3 class 2 COMPOUNDS, and all 6 class 3 COMPOUNDS achieved similarity targets 1 and 2.

Discussion

The idea presented is simple: build an ISHC software device that, during simulation, mimics essential, abstract features of hepatocytes in culture interacting with, transporting, and clearing xenobiotics metabolically. Complicated mechanisms can be subdivided into simpler, networked micromechanisms. By so doing, the mapping from PCP space to micromechanism parameterizations is simplified and more easily predicted. The relative dependence of COMPOUND clearance properties within ISHCs on PCPs is intended to map to the relative dependence observed in vitro over a wide variety of PCPs. ISHC events and influence scores were simple because an operating hypothesis was that the variability of the in vitro counterpart, across many xenobiotics, can be accounted for primarily by intercompound variability in a small set of influential PCPs. An event-specific influence score function is a placeholder for the more detailed mechanistic knowledge that will replace it eventually as it accumulates. A limitation of the ISHC approach at this early stage is that there probably exists a large, constrained set of equally effective (in achieving SM targets) influence score functions for each event in Fig. 1. We specified and used just one per event. We hypothesize that development and challenge of other influence score functions using expanded data sets combined with additional targeted attributes will lead to achievement of more stringent SM targets. The process of allowing many different influence score functions to compete with each other (for improving SMs) can be automated, although we have not yet done so.

There are also many options for translating influence score values into ISHC parameter values. Again, we used one. It enabled achievement of a degree of pCL_{is} and CL similarity. Others, including methods tailored to each event, must be explored to achieve more stringent SM targets.

Although ISHC prediction performance was better than that of multiple linear regression for the subset of 39 compounds for which S.D. values were available, the prediction performances for the two methods for the full set of 73 compounds were similar: both were poor. A contributing factor to the latter was that the pCL_{is} range in Table 3 (0.35–1.44), was too narrow: CL values ranged from 0.2 to 107 $\mu l \cdot min^{-1} \cdot 10^6\ cells^{-1}$ (0.2–37 if the two largest values are ignored). Nevertheless, given the simple and abstract nature of ISHCs, it is noteworthy that ISHC predictions performed as well as they did. It is clear that improvement is needed, and the IR protocol provides a means of achieving it by making ISHCs incrementally more complicated or fine-grained on an as-needed basis. Our goal is that CL_{is} and CL be directly correlated. Given that, one approach to expand the CL_{is} range is to enable ISHCs to "run" faster or slower, depending on selected PCPs. So doing can accommodate experimenting on multiple COMPOUNDS simultaneously. At present, everything is updated once per simulation cycle for all COMPOUNDS. We can provide a COMPOUND-dependent option for updating *n* times per simulation cycle. There are other options. For example, in a somewhat more complicated approach, we can change the stochasticity of one or more ISHC events in a PCP-dependent fashion. Current event occurrence (or not) is based on comparison of the stochastic parameter value to a number

drawn randomly from a uniform distribution. We can bias the draws by using a distribution having an explicitly manipulable variance that is PCP-determined, as done in Park et al. (2009). The pros and cons of these and other options can be explored in parallel within future, competing ISHCs.

For simplicity in demonstrating concept feasibility, we specified that INTRACELLULAR and EXTRACELLULAR nonspecific binding within ISHCs be the same for all COMPOUNDS. Moving forward, we need to eliminate that simplification. We envision exploring several nonspecific binding influence score functions. In the absence of a method to select one over the other, we can use several: each simulation cycle, select one at random from the set and use it to parameterize the binding events for that COMPOUND for that cycle. So doing would increase the variability within and between simulation experiments, which itself has scientific merit: it builds identifiable uncertainties into ISHC simulations. Averaging results from more simulation experiments would provide whatever precision is needed.

The targeted attribute for this study was simply CL. A path moving forward should include additional attributes. We can begin with specific subgroups of compounds, for example. In addition to achieving specific pCL_{is} targets, we might require that the relative rank of pCL_{is} values within a subgroup be the same and quantitatively similar to the relative rank of corresponding CL values. Acebutolol, atenolol, betaxolol, metoprolol, nadolol, pindolol, and propranolol are all β-blockers (same pharmacological class). Their CL values range from large (propranolol) to very small (atenolol). Making this attribute a required target would falsify the current ISHC. We would need to cycle through the IR protocol several times until the new, targeted attributes are achieved.

In vitro experimental variability is a well recognized problem (Baker and Parton, 2007; Stringer et al., 2008; Hallifax et al., 2010; Huang et al., 2010; Rostami-Hodjega, 2010). The approach presented offers a new strategy for managing some aspects of the problem. ISHCs that have achieved a degree of validation against CL values measured in one in vitro system can be reused for another. When two or more compounds are common to both sets, which is often the case, we can explore component adjustments (e.g., changing the relative number of ENZYMES and TRANSPORTERS) of one ISHC so that micromechanisms for the common compounds are similar in both systems. By so doing, we have a concrete hypothesis about sources of variability. As demonstrated with in silico normal and diseased livers (Park et al., 2010), it is straightforward to morph one in silico system into another. So doing provides a testable hypothesis for how, where, and why the two in vitro systems are different.

ISHCs were designed deliberately to enable the relative kinetics of different events for different COMPOUNDS to map quantitatively to the relative kinetics of their compound counterparts in hepatocyte cultures. However, the actual kinetics during ISHC simulations do not map quantitatively to actual kinetic counterparts in hepatocyte cultures. That difference is a combined consequence of insisting on relational rather than absolute grounding and of how ISHCs and COMPOUNDS were discretized. If we were to insist that the kinetics of COMPOUNDS map quantitatively to hepatocyte culture counterparts (which imposes a particular type of absolute grounding between ISHC and referent), then, in the limit, the model would need to be just as complicated as the referent. We would no longer be seeking a model; our objective would be a detailed, accurate in silico "copy" of events and processes within the in vitro system. As explained in Hunt et al. (2009), such absolute grounding would only be scientifically useful under conditions identical to those under which referent wet laboratory experiments have been conducted.

Using multiple linear regression as a standard against which to judge ISHC performance is a reasonable first step. More sophisticated regression methods, including those that exclude negative values, may enable CL predictions that equal or exceed current SM values. The expectation is that having the ability to easily revise ISHCs and instantiate improved, validated, mechanistic knowledge will enable predictions from future ISHCs to perform better than correlation-based prediction methods. That is in part because more of what we think we know about PCP-CL relationships will be built into ISHC mechanisms and automated parameterization protocols. In some cases, inductive methods such as artificial neural networks (Paixão et al., 2010) may still be able to outperform ISHCs, but they are not mechanism-based.

A contribution of this work is offering a method for combining both the knowledge of mechanisms with related knowledge about patterns found in the space of PCPs and reported CL values. Our approach leverages that knowledge by representing and improving our understanding of the generative relationships within hepatocytes that are responsible for measured CL values. The generative relationships between components within ISHCs stand as a hypothesis of how clearance phenomena are being generated. As such, the approach presented herein is expected to significantly improve our ability to anticipate the clearance properties of new compounds. Enabling prediction of in vivo hepatic clearance in humans will require plugging ISHCs into validated in silico livers (Park et al., 2009, 2010) within larger patient analogs constructed and validated using these same scientific methods of modeling and simulation.

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Authorship Contributions

Participated in research design: Sheikh-Bahaei and Hunt.
Conducted experiments: Sheikh-Bahaei.
Contributed new reagents or analytic tools: Sheikh-Bahaei.
Performed data analysis: Sheikh-Bahaei.
Wrote or contributed to the writing of the manuscript: Sheikh-Bahaei and Hunt.

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